Virological studies on foot and mouth disease in sheep

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SUMMARY

74 (56 epithelial flaps – 18 nasal swabs) samples were collected from clinically diseased sheep in two private farms belonging to Sekum company, Sharkia Governorate and other private farm at Cairo – Alexandria Desert Road during 2008. In addition 105 serum samples were collected from diseased and contact sheep in the same farms. Vesicular flaps and nasal swabs were used for FMD antigen detection by indirect sandwich Enzyme Linked Immuno Sorbant Assay (ELISA) where twenty one out of 56 vesicular flaps (37.5%) were positive for FMD serotype O and negative for FMD serotype A. In contrast, all nasal swabs were negative. FMD positive samples by ELISA were propagated on Baby Hamster Kidney (BHK21) cell line. Clear cytopathic Effect (CPE) was developed after third blind passage for only 12 isolates. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) assay was applied as confirmatory advanced technique on the harvest of infected cell culture to confirm and identify the positive isolates.

Serum Neutralization Test (SNT) was used for detection of FMD virus antibodies against FMDV serotype O & A in serum sample where 62 out of 105 (59%) were positive for serotype O and all negative for presence of antibodies for serotype A.

INTRODUCTION

Foot and mouth disease (FMD) is a highly contagious viral disease of cloven – hoofed domesticated and wild animals. Swine, Cattle, Sheep and Goats are most susceptible. Animals infected with FMDV will typically develop a fever followed by formation of vesicles and erosions in the mucosa of the mouth, tongue, lips, soft plate, nostril, teats, gums, dental pads and the skin of the inter digital spaces (Radostitis et al., 1995). These vesicles rupture soon after they develop resulting in the exposure of raw sensitive tissue underneath. Lesions of the oral cavity cause excessive salivation and make eating painful so animal will go "off
Lesions on the feet cause lameness so animals are reluctant to move and often prefer to lie down. It has detrimental effects on meat and milk production (Radosstitis et al., 1995).

FMD is caused by Picorna virus, of genus Aphthovirus. It contains a single stranded RNA molecule and has seven major serotypes: A, O, C, SAT1, SAT 2, SAT3 and ASIA 1. Infection with one serotype does not confer immunity against another (OIE Manual, 2005). Recovered animals or these products are considered most important source of the virus and can be mean of spread. Therefore, livestock or animal products from countries with FMD are not welcome in FMD-free countries. As a result, the disease has serious economic trade impact (Anthony and Werner, 1992).

FMD virus has 4 structural proteins (SP) (VP1, VP2, VP3 and VP4) forming the capsid and when it replicates infection, results in the production of a numbers of non-structural proteins, (NSP) of which some are immunogenic.

The importation of live animals from some African countries as a trade cooperation between Egypt and African countries is considered the corner stone of rethinking of new epizootic, that may introduced with the imported animals (Samuel and Knowles, 2001). In Egypt, FMD was first detected in 1950 when strain SAT2 caused an outbreak, then in 1952, 1956 and 1958 when outbreaks were caused by strain A, where several foci were detected in years 1961, 1962, 1964, 1965, 1966, 1967, 1968, 1969 and 1970. No any strains of FMD other than O have been detected since 1970. During the year 2006, FMD has taken an enzootic form in Egypt caused by the new exotic strain of FMD virus serotype (A), where susceptible animals showed more severe forms than others caused by dominant serotype O (Nawal et al., 2006).

The need to diagnose FMD is of paramount importance in all countries even in those countries where it occurs enzootically. Also, it is of particular importance in those countries which do not experience the disease because of the need to introduce control measures quickly (Blood et al., 1983).

The present study aimed to determine the causative agent of this problem (appearance of signs on sheep suspected to FMD in the two farms) by antigen detection through Indirect sandwich Enzyme Linked Immuno Sorbant assay (ELISA) then virus isolation on tissue culture and identification of the isolates. Reverse Transcription- Polymerase Chain Reaction (RT – PCR) was also applied to confirm and identify the isolated strains. In addition, determination
of antibodies against FMDV serotypes A & O on sera sample of diseased and contact sheep was done by Serum Neutralization Test (SNT).

MATERIAL AND METHODS
1- Samples:

Table (1): Samples collected from clinically diseased and contact sheep of the two private farms at Sharkia and Alexandria Governorates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sekum (Sharkia)</th>
<th>Cairo – Alexandria Desert Road</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicular flaps</td>
<td>39</td>
<td>17</td>
<td>56</td>
</tr>
<tr>
<td>Nasal swabs</td>
<td>18</td>
<td>---</td>
<td>18</td>
</tr>
<tr>
<td>Serum</td>
<td>71</td>
<td>34</td>
<td>105</td>
</tr>
</tbody>
</table>

56 samples of vesicular flaps of the tongue, gum in addition to 18 nasal swabs were collected on glycerol buffer saline for antigen detection, serotyping determination and isolation of the causative viral agent as well as confirmation and identification by RT-PCR technique during 2008.

105 serum samples were collected from diseased and contact sheep for determination of antibodies against FMDV serotypes A&O by SNT.

2- Cell culture:
Baby hamster kidney (BHK 21) cell line was obtained from the Egyptian Organization for Biological and Vaccine Production, Cairo, Egypt. The cells were grown using 10% FCS in media and maintained with 2% FCS in media, in addition to using antibiotics.

3-Virological investigation:
3-1. Antigen detection and serotyping determination:
All collected samples for antigen detection and serotyping determination (Serotypes A&O) were tested by indirect sandwich ELISA according to the protocol of OIE / EAO WRLab for FMD, Pirbright, UK and to Hamblin et al. (1986 a&b).

3-2. Isolation and identification of the causative agent:
The positive samples for FMD antigen detection and sero-
typing determination by ELSA were propagated on BHK21 cell line for three blind passages and then identified by VNT according to OIE (2005) and Clarke and Spire (1980).

4- Molecular confirmation:
Reverse transcription Polymerase chain reaction (RT-PCR):
It was successfully done on positive isolates which revealed clear cytopathic effect on tissue culture and the procedures used was previously described by Reid et al. (1999) and (2000).

* RNA extraction:
Total RNA was extracted from the positive control and the sample with TRizol Reagent (Life Technologies, UK).

* Reverse transcription:
RNA was subjected to reverse transcription using random primers at 37 C for 45 min in 20-ul reaction volume as described previously Reid et al. (2000).

* PCR mix:
Nuclease-free water (35 μl); PCR reaction buffer, 10 x conc (5 μl); MgCl₂, 50 mM (1.5 μl); dNTPs, 10mM mixture each of dATP, dCTP,dGTP,dTTP(1μl); primer1 (5'-GCCTG-GTCTT-TCCAG-GTCT-3') 10 pmol/μl (1 μl); primer2 (5'CCAGT-

5- Serum Neutralization Test (SNT):
It was applied on serum samples according to Edwin and Nathalic (1979) using FMDV serotype A &O which obtained from virology research department, Animal health Institute. Dokki, Giza.
RESULTS

Table (2): FMD antigen detection and serotyping determination (serotypes A&O) by indirect sandwich Enzyme Linked Immuno Sorbant Assay (ELISA).

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Samples No.</th>
<th>Epithelial flaps</th>
<th>Nasal swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve -ve</td>
<td>+ve -ve</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No %</td>
<td>No %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serotype A</td>
<td>Serotype O</td>
<td>Serotype A</td>
</tr>
<tr>
<td></td>
<td>No No</td>
<td>No No</td>
<td>No No</td>
</tr>
<tr>
<td>Sharkia</td>
<td>39 15</td>
<td>61 7</td>
<td>18 18 18</td>
</tr>
<tr>
<td>Alexandria</td>
<td>17 6</td>
<td>64 7</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>56 21</td>
<td>67 5</td>
<td>18 18 18</td>
</tr>
</tbody>
</table>

N.B. All collected samples (epithelial flaps and nasal swabs were negative for serotype A.

Table (3): Number of positive ELISA samples resulted clear cytopathic Effect (CPE) on (BHK21) cell line after the third blind passage.

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Total samples</th>
<th>Positive ELISA samples</th>
<th>Positive clear CPE</th>
<th>% of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sharkia</td>
<td>39</td>
<td>15</td>
<td>9</td>
<td>60</td>
</tr>
<tr>
<td>Alexandria</td>
<td>17</td>
<td>6</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>21</td>
<td>12</td>
<td>57.1</td>
</tr>
</tbody>
</table>
**Table (4):** Screening of FMD virus antibodies against FMD serotype A&O in serum samples by Serum neutralization test (SNT).

<table>
<thead>
<tr>
<th>Governorate</th>
<th>Serum samples</th>
<th>Serotype O</th>
<th></th>
<th></th>
<th>Serotype A</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of +ve</td>
<td>%</td>
<td>No. of +ve</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sharkia</td>
<td>71</td>
<td>42</td>
<td>59.2</td>
<td>11</td>
<td>15.5</td>
<td></td>
</tr>
<tr>
<td>Alexandria</td>
<td>34</td>
<td>20</td>
<td>58.8</td>
<td>6</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>62</td>
<td>59</td>
<td>17</td>
<td>16.2</td>
<td></td>
</tr>
</tbody>
</table>

NB: titer of +ve samples were ranged between 1/16 – 1/32

The result of molecular confirmation on the positive samples which gave clear and characteristic cytopathic effect by **RT-PCR** revealed that the positive result of RT-PCR by the presence of a **338 bp** band. Their positivity indicated by the presence of 338 bp band in where the PCR products were electrophoresed (Fig., 2)

Fig. (1): Showed ulcers on the gum and mouth cavity revealed from the effect FMD (x g 400).
DISCUSSION

Foot-and-Mouth Disease (FMD) is a notifiable disease in most countries of the world, and any clinical suspicion of diseases should be reported to the appropriate authorities. The disease occurs in most of the major livestock producing countries of the world, except North America, Central America, Australia, New Zealand, Japan and Ireland.

The disease is characterized by the formation of vesicles and erosions in the mucosa of the mouth, external nares in coronary band of claws of the feet; other areas, including udder and teats. Lameness is seen, reduced lactation, mastitis and abortion are common (OIE-Manual, 2005).

Recognized picture of FMD clinical signs was reported in January 2006. The affected cattle suffered from fever associated with depression, anorexia, loss of appetite, lacrimation, and excessive salivation, serous nasal discharge, and mouth and foot lesions which was attributed to exotic FMDV serotype A. Followed these outbreak, many foci frequently were appeared with long or short intervals (Nawal et al., 2006). In 2008 the
disease was appeared in cattle again but in the places of our studies the disease was started in sheep which showed a clinical pictures of the disease with varies stages and then was transmitted to cattle (Fig., 1).

The role of sheep in maintenance of FMD virus remains uncertain, so the design of appropriate disease control measures is problematic. In particular, it is not proven that prophylactic vaccination program of small ruminants is essential to eradicate the disease and there is some evidence that FMD in sheep is self limiting. Owing to the cost of vaccination, small ruminants still not to be routinely vaccinated unless there is an association with large number of cattle.

In spite of high contagious nature of FMD and the complicated mode of infection which depends mainly on aerosolic transmission and direct contact with infected animals. In addition to the high susceptibility of sheep to FMD, this study was planed to focus more light on the causative agent of this problem by antigen detection and serotyping of FMDV by Indirect sandwich Enzyme Linked Immuno Sorbant assay (ELISA) and virus isolation on tissue culture. Table (2) showed that the positive percentage of ELISA on epithelial flaps was in range of 37% in the two farms, and these findings are in agree with (Nawal et al. 2006 and Hambline et al., 1986 a, b) in addition to all nasal swabs were negative to ELISA.

Isolation and identification of FMDV, table (3) revealed that 60% of positive ELISA samples resulted clear cytopathic Effect (CPE) on (BHK21) cell line after the third blind passage in Sharkia and 50% in Alexandria with average 55% .then identified by SNT, where these results are in agree with which mentioned by OIE Manual (2005) and Clarke and Spire (1980).

Concerning the screening of FMDV serotype O&A, Table (4) showed the results of determination of antibodies against FMDV serotype O, on sera sample of diseased and contact animals by Serum Neutralization Test (SNT) with percentage 59.2% and 58.8% in Sharkia and Alexandria respectively. In contrast , with percentage 15.5% and 17.6% in Sharkia and Alexandria respectively with titer lower than 1/8 these results attributed to the stopping of vaccination of sheep , goat and camel with FMD vaccines at January 2008, these stopping of vaccination lead to presence of sheep and goat as source of infection . These results are in agreed with those described by Edwin and Nathalic (1979) and Payment et al. (1993).
In addition, the successfully application of Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for more confirmation and identification revealed that all positive samples for isolation were also positive for RT-PCR, hence, RT-PCR was proved that it is accurate, rapid, specific, sensitive and modern technique which have to applied with large scale in veterinary diagnosis. The results of RT-PCR were the same done by (Reid et al. 2000 & 2002). Fig (2)

From the previous results, sheep and goat showed very important role in the epidemiology of FMD in Egypt. It has to highlight on their role in the skeleton of the epidemiological map of FMD.

Hence, our recommendations to reform the vaccination policy in Egypt especially for sheep and goat and return the vaccination programme for sheep with cattle, where the nature of rearing animals in Egypt mostly together

REFERENCES


OIE (2005): manual of Diagnostic tests and vaccines for terrestrial animals.


Reid, S.M.; Ferris, N.P.; Hutchings, G.;; Zhang, Z.; and